

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/10, 15/87, 15/88, C07H 21/04	A1	(11) International Publication Number: WO 00/04139 (43) International Publication Date: 27 January 2000 (27.01.00)
(21) International Application Number: PCT/US99/16107 (22) International Filing Date: 16 July 1999 (16.07.99) (30) Priority Data: 60/093,231 17 July 1998 (17.07.98) US (71) Applicant: MIRUS CORPORATION [US/US]; 545 Science Drive, Madison, WI 53711 (US). (72) Inventor: WOLFF, Jon, A.; 1122 University Bay Drive, Madison, WI 53705 (US). (74) Agent: JOHNSON, Mark, K.; P.O. Box 510644, New Berlin, WI 53151-0644 (US).		(81) Designated States: JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: MICELLAR SYSTEMS (57) Abstract A complex is described for delivery to a cell comprising inserting a nucleic acid into a reverse micelle. The reverse micelle has the property to compact the nucleic acid for easier delivery. Other molecules are used to interact with the nucleic acid-micelle complex to further enhance delivery such as a surfactant having a disulfide bond.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Micellar Systems

Field of the Invention

5

The invention generally relates to micellar systems for use in biologic systems. More particularly, a process is provided for the use of reverse micelles for the delivery of nucleic acids and genes to cells.

10

Background

Biologically active compounds such as proteins, enzymes, and nucleic acids have been delivered to the cells using amphipathic compounds that contain both hydrophobic and hydrophilic domains. Typically these amphipathic compounds are organized into vesicular structures such as liposomes, micellar, or inverse micellar structures.

15 Liposomes can contain an aqueous volume that is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids) (R.C. New, p. 1, chapter 1, "Introduction" in Liposomes: A Practical Approach, ed. R.C. New IRL Press at Oxford University Press, Oxford, 1990). Micelles and inverse micelles are microscopic
20 vesicles that contain amphipathic molecules but do not contain an aqueous volume that is entirely enclosed by a membrane. In micelles the hydrophilic part of the amphipathic compound is on the outside (on the surface of the vesicle) whereas in inverse micelles the hydrophobic part of the amphipathic compound is on the outside. The inverse micelles thus contain a polar core that can solubilize both water and macromolecules
25 within the inverse micelle. As the volume of the core aqueous pool increases the aqueous environment begins to match the physical and chemical characteristics of bulk water. The resulting inverse micelle can be referred to as a microemulsion of water in oil (Schelly, Z. A. Current Opinion in Colloid and Interface Science, 37-41, 1997; Castro, M. J. M., Cabral, J. M. S. Biotech. Adv. 6, 151-167, 1988).

30

Microemulsions are isotropic, thermodynamically stable solutions in which substantial amounts of two immiscible liquids (water and oil) are brought into a single phase due to a surfactant or mixture of surfactants. The spontaneously formed colloidal particles are globular droplets of the minor solvent, surrounded by a monolayer of surfactant

molecules. The spontaneous curvature, H_0 of the surfactant monolayer at the oil/water interface dictates the phase behavior and microstructure of the vesicle. Hydrophilic surfactants produce oil in water (O/W) microemulsions ($H_0 > 0$), whereas lipophilic surfactants produce water in oil (W/O) microemulsions. When the hydrophile-lipophile
5 properties of the surfactant monolayer at the water/oil interface are balanced bicontinuous-type microemulsions are formed ($H_0 = 0$).

Positively-charged, neutral, and negatively-charged liposomes have been used to deliver nucleic acids to cells. For example, plasmid DNA expression in the liver has
10 been achieved via liposomes delivered by tail vein or intraportal routes. Positively-charged micelles have also been used to package nucleic acids into complexes for the delivery of the nucleic acid to cells. Negatively-charged micelles have been used to condense DNA, however they have not been used for the delivery of nucleic acids to cells (Imre, V. E., Luisi, P. L. Biochemical and Biophysical Research Communications,
15 107, 538-545, 1982). This is because the previous efforts relied upon the positive-charge of the micelles to provide a cross-bridge between the polyanionic nucleic acids and the polyanionic surfaces of the cells. Micelles that are not positively-charged, or that do not form a positively charged complex cannot perform this function. For example, a recent report demonstrated the use of a cationic detergent to compact
20 DNA, resulting in the formation of a stable, negatively-charged particle (Blessing, T., Remy, J. S., Behr, J. P. Proc. Natl. Acad. Sci. USA, 95, 1427-1431, 1998). A cationic detergent containing a free thiol was utilized which allowed for an oxidative dimerization of the surfactant to the disulfide in the presence of DNA. However, as expected, the negatively-charged complex was not effective for transfection.

25 Reverse (water in oil) micelles has also been used to make cell-like compartments for molecular evolution of nucleic acids (Tawfik, D.S. and Griffiths, A.D. Nature Biotechnology 16:652, 1998). Cleavable micellar systems was not used in this system.

30 In addition, Wolff et al. have developed a method for the preparation of DNA/amphipathic complexes including micelles in which at least one amphipathic compound layer that surrounds a non-aqueous core that contains a polyion such as a nucleic acid (Wolff, J., Budker, V., and Gurevich, V. U.S. Patent # 5635487).

Cleavable Micelles

A new area in micelle technology involves the use of cleavable surfactants to form the micelle. Surfactants containing an acetal linkage, azo-containing surfactants, elimination of an ammonium salt, quaternary hydrazonium surfactants, 2-alkoxy-N,N-dimethylamine N-oxides, and ester containing surfactants such as ester containing quaternary ammonium compounds and esters containing a sugar have been developed.

These cleavable surfactants within micelles are designed to decompose on exposure to strong acid, ultraviolet light, alkali, and heat. These conditions are very harsh and are not compatible with retention of biologic activity of biologic compounds such as proteins or nucleic acids. Thus, biologically active compounds have not been purified using reverse micelles containing cleavable surfactants.

Complexation of Nucleic Acids with Polycations

Polymers are used for drug delivery for a variety of therapeutic purposes. Polymers have also been used for the delivery of nucleic acids (polynucleotides and oligonucleotides) to cells for therapeutic purposes that have been termed gene therapy or anti-sense therapy. One of the several methods of nucleic acid delivery to the cells is the use of DNA-polycation complexes. It was shown that cationic proteins like histones and protamines or synthetic polymers like polylysine, polyarginine, polyornithine, DEAE dextran, polybrene, and polyethylenimine were effective intracellular delivery agents while small polycations like spermine were ineffective. As a result the main mechanism of DNA translocation to the intracellular space might be non-specific adsorptive endocytosis which may be more effective than liquid endocytosis or receptor-mediated endocytosis. Furthermore, polycations are a very convenient linker for attaching specific receptors to DNA and as result, DNA-polycation complexes can be targeted to specific cell types.

There are a variety of molecules (gene transfer enhancing signals) that can be covalently attached to the gene in order to enable or enhance its cellular transport.

These include signals that enhance cellular binding to receptors, cytoplasmic transport to the nucleus and nuclear entry or release from endosomes or other intracellular vesicles.

- 5 Nuclear localizing signals enhance the entry of the gene into the nucleus or directs the gene into the proximity of the nucleus. Such nuclear transport signals can be a protein or a peptide such as the SV40 large T ag NLS or the nucleoplasmin NLS.

- Other molecules include ligands that bind to cellular receptors on the membrane
10 surface increasing contact of the gene with the cell. These can include targeting group such as agents that target to the asialoglycoprotein receptor by using asialoglycoproteins or galactose residues. Other proteins such as insulin, EGF, or transferrin can be used for targeting. Peptides that include the RGD sequence can be used to target many cells. Chemical groups that react with sulfhydryl or disulfide
15 groups on cells can also be used to target many types of cells. Folate and other vitamins can also be used for targeting. Other targeting groups include molecules that interact with membranes such as fatty acids, cholesterol, dansyl compounds, and amphotericin derivatives.

- 20 Size of a DNA complex may be a factor for gene delivery in vivo. Many times, the size of DNA that is of interest is large, and one method of delivery utilizes compaction techniques. The DNA complex needs to cross the endothelial barrier and reach the parenchymal cells of interest. The largest endothelia fenestrae (holes in the endothelial barrier) occur in the liver and have average diameter of 100 nm. The trans-epithelial
25 pores in other organs are much smaller, for example, muscle endothelium can be described as a structure which has a large number of small pores with a radius of 4 nm, and a very low number of large pores with a radius of 20-30 nm.(Rippe, B. Physiological Rev, 1994). The size of the DNA complex is also important for the cellular uptake process. After binding to the target cells the DNA complex should be
30 taken up by endocytosis. Since the endocytic vesicles have a homogenous internal diameter of about 100 nm in hepatocytes, and are of similar size in other cell types, the DNA is compacted to be smaller than 100 nm.

Compaction of DNA

There are two major approaches for compacting DNA:

1. Multivalent cations with a charge of three or higher have been shown to condense
5 DNA. These include spermidine, spermine, $\text{Co}(\text{NH}_3)_6^{3+}$, Fe^{3+} , and natural or synthetic
polymers such as histone H1, protamine, polylysine, and polyethylenimine. One
analysis has shown DNA condensation to be favored when 90% or more of the charges
along the sugar-phosphate backbone are neutralized (Wilson, R. W., Bloomfield, V. A.
Biochemistry 18, 2192-2196, 1979).
- 10 2. Polymers (neutral or anionic) which can increase repulsion between DNA and its
surroundings have been shown to compact DNA. Most significantly, spontaneous
DNA self-assembly and aggregation process have been shown to result from the
confinement of large amounts of DNA, due to excluded volume effect (Strzelecka, T.
E., Rill, R. L. Biopolymers 30, 803-14, 1990; Strzelecka, T. E., Rill, R. L.
15 Biopolymers 30, 57-71, 1990). Since self-assembly is associated with locally or
macroscopically crowded DNA solutions, it is expected, that DNA insertion into small
water cavities with a size comparable to the DNA will tend to form mono or
oligomolecular compact structures.

20

Micelles and Reverse Micelles

- Reverse micelles (water in oil microemulsions) are widely used as a host for
biomolecules. Examples exist of both recovery of extracellular proteins from a culture
broth and recovery of intracellular proteins. Although widely used, recovery of the
25 biomolecules is difficult due to the stability of the formed micelle and due to
incomplete recovery during the extraction process. Similarly, purification of DNA or
other biomolecules from endotoxin and plasma is difficult to accomplish. One
common method employing Triton results in incomplete separation of the DNA or
biomolecules from the emulsion.

30

Reverse micelles have been widely used as a host for enzymatic reactions to take place.
In many examples, enzymatic activity has been shown to increase with the used in a
micelle, and has allowed enzymatic reactions to be conducted on water insoluble

substrates. Additionally, enzymatic activity of whole cells entrapped in reverse micelles has been investigated (Gajjar, L., Singh, A., Dubey, R. S., Srivastava, R. C. Applied Biochemistry and Biotechnology, 66, 159-172, 1997). The cationic surfactant cetyl pyridinium chloride was utilized to entrap Baker's yeast and Brewer's yeast inside
5 a reverse micelle.

Micelles have also been used as a reaction media. For example, a micelle has been used to study the kinetic and synthetic applications of the dehydrobromination of 2-(p-nitrophenyl)ethyl bromide. Additionally, micelles have found use as an emulsifier for emulsion polymerizations.

10

Micelles have been utilized for drug delivery. For example, an AB block copolymer has been investigated for the micellar delivery of hydrophobic drugs. Transport and metabolism of thymidine analogues has been investigated via intestinal absorption utilizing a micellar solution of sodium glycocholate. Additionally, several examples of
15 micelle use in transdermal applications have appeared. For example, sucrose laurate has been utilized for topical preparations of cyclosporin A.

Summary

20 The present invention provides for the transfer of polynucleotides, and biologically active compounds into parenchymal cells within tissues in situ and in vivo, utilizing reverse micelles delivered intravascularly, intrarterially, intravenous, orally, intraduodenally, via the jejunum (or ileum or colon), rectally, transdermally, subcutaneously, intramuscularly, intraperitoneally, intraparenterally, via direct
25 injections into tissues such as the liver, lung, heart, muscle, spleen, pancreas, brain (including intraventricular), spinal cord, ganglion, lymph nodes, lymphatic system, adipose tissues, thyroid tissue, adrenal glands, kidneys, prostate, blood cells, bone marrow cells, cancer cells, tumors, eye retina, via the bile duct, or via mucosal membranes such as in the mouth, nose, throat, vagina or rectum or into ducts of the
30 salivary or other exocrine glands.

By "delivered" we mean that the polynucleotide becomes associated with the cell. The polynucleotide can be on the membrane of the cell or inside the cytoplasm, nucleus, or

other organelle of the cell. The process of delivering a polynucleotide to a cell has also been commonly termed "transfection" or the process of "transfecting" and also it has been termed "transformation". The polynucleotide could be used to produce a change in a cell that can be therapeutic. The delivery of polynucleotides or genetic material for therapeutic purposes is commonly called "gene therapy". The polynucleotides or genetic material being delivered are generally mixed with transfection reagents prior to delivery. A biologically active compound is a compound having the potential to react with biological components. Pharmaceuticals, proteins, peptides, hormones, cytokines, antigens and nucleic acids are examples of biologically active compounds. The reverse micelle may be negatively-charged, zwitterionic, or neutral. Additionally, the present invention provides for the purification of biomolecules by solubilizing the biomolecule into a cleavable reverse micelle and then cleaving the micelle under conditions that will not destroy the biological activity of the biomolecule. These processes can be used for transferring nucleic acids or biomolecules into cells or an organism such as for drug delivery, or may also be used for analytical methods.

The process of utilizing cleavable reverse micelles for the purification of biomolecules has advantages over current methodology. Isolation of the biomolecule will be enhanced by cleaving the reverse micelle. This will separate the polar group from the non-polar group on the surfactant and therefore eliminate the formation of emulsions and therefore simplify the isolation process. Cleavage conditions will be such that the biological activity of the biomolecule is not destroyed.

Another advantage of the invention is the use of reverse micelles for gene delivery. The reverse micelle can compact the polynucleotide, a critical step for gene delivery, especially in vivo. The micelle containing the compacted polynucleotide can then be utilized as a reaction vesicle in which additional compounds can be added to the DNA. For example, a polycation could be added to the polynucleotide/reverse micelle solution to form a polycation/polynucleotide complex within the reverse micelle. Additionally, the polynucleotide/reverse micelle system is used as a vesicle for template polymerization of the DNA or caging of the DNA in which the polycation is crosslinked. A variety of chemical reactions can take place within the vesicle preferably without modifying the nucleic acid. The polynucleotide / reverse micelle

system also has advantages in that the micelle may be cleaved under physiological conditions involved along the "transfection pathway." The surfactant can be altered so that micellular cleavage occurs at different point along this pathway. By "transfection pathway" we mean any point at which the polynucleotide/reverse micelle system is introduced to a solution (i.e., blood, serum) that contains parenchymal cells, or to the cells (for example the skin or mucousal membranes) through the inclusion of the polynucleotide into the nucleus of the parenchymal cell.

In a preferred embodiment, described is a complex for delivery to a cell, comprising: inserting a nucleic acid into a reverse micelle. A compacting agent may be added to the complex as well as a delivery enhancing ligand or compound.

In a preferred embodiment, a process for delivering a complex to a cell is described, comprising inserting a nucleic acid into a reverse micelle.

In a preferred embodiment, the nucleic acid or biomolecule is solubilized into a reverse micelle with an internal water volume for delivery of the biomolecule to parenchymal cells. A compound can be added to the nucleic acid/micelle mixture. Such compounds include polymers such as polyions (polycations such as spermine, polyamines, polylysine, polyethylimine (PEI), and polyanions), proteins, peptides, enzymes, hydrophobic compounds, and amphipathic compounds (to form a second layer around the micelle). Such compounds include compounds that compact the DNA, provide a cell transfer enhancing ligand or provide another layer to the micelle.

In another preferred embodiment, the nucleic acid or biomolecule is complexed with another molecule prior to micelle formation. For example, a polymer such as polylysine is added to the nucleic acid and then crosslinked to cage the nucleic acid. When a nucleic acid is caged a polymer is formed since the polylysine (or any type of polymer) acts as a monomer that is being included in another polymer.

Another preferred embodiment provides a method of making a compound for delivery to a cell, comprising: adding one or more compounds to the nucleic acid or biomolecule/reverse micelle complex prior to delivery to the cell, thereby providing a

deliverable complex. For example, another surfactant or a polyion might be added to the complex. The cell can be a prokaryote or eukaryote and can be a plant, animal or mammalian cell.

- 5 Another preferred embodiment provides a method of making a compound for delivery to a cell, comprising: modifying a molecule in the presence of the biomolecule/reverse micelle complex thereby providing a deliverable complex.

10 In another preferred embodiment, the parenchymal cell is solubilized within a reverse micelle. A reverse micelle containing a polynucleotide would be added to the parenchymal cell containing reverse micelle. After an appropriate amount of time, the parenchymal cell would be purified, and delivered to a mammal.

15 In another preferred embodiment the biomolecule is solubilized utilizing one of the following procedures.

Procedure 1.

- a) mixing of the biomolecule into an aqueous solution
- b) then mixing the aqueous solution containing the biomolecule with a hydrocarbon or haloalkane containing a surfactant with agitation or sonication

20 Procedure 2.

Mixing of biomolecule into a solution containing a reverse micelle with agitation or sonication.

Procedure 3.

- a) mixing the biomolecule into an aqueous solution
- 25 b) then extracting the aqueous solution containing the biomolecule with a hydrocarbon or haloalkane containing a reverse micelle and separating the phases.

30 In another preferred embodiment, the biomolecule is purified comprising a step in which a reverse micelle is destroyed.

In another preferred embodiment, the biomolecule can be purified by utilizing one of the following procedures.

Procedure 1.

- a) mixing of the biomolecule into an aqueous solution
- b) then mixing the aqueous solution containing the biomolecule with a hydrocarbon or halohydrocarbon containing a surfactant with agitation or
5 sonication
- c) cleaving the reverse micelle
- d) extract the biomolecule

Procedure 2.

- a) mixing of biomolecule into a solution containing a reverse micelle with
10 agitation or sonication.
- b) cleaving the reverse micelle
- c) extract the biomolecule

Procedure 3.

- a) mixing the biomolecule into an aqueous solution
- 15 b) then extracting the aqueous solution containing the biomolecule with a hydrocarbon or halohydrocarbon containing a reverse micelle and separating the phases.
- c) cleaving the reverse micelle
- d) extract the biomolecule

20

In another preferred embodiment, the surfactant is a disulfide of general formula A-S-S-B, which contains a hydrophobic group (A) and a hydrophilic group (B).

25 In another preferred embodiment, the surfactant can be cleaved in the presence of the nucleic acid or biomolecule under conditions that will not destroy the biological activity of the nucleic acid or biomolecule.

In another preferred embodiment, the surfactant could be chemically modified in the presence of the nucleic acid or biomolecule. For example, the surfactant can be
30 polymerized after micelle formation to form a "shell" or cage around the nucleic acid. The surfactant could be cleaved separating the bulk of its hydrophilic and hydrophobic parts thus destroying its ability to act as a surfactant.

In yet another preferred embodiment, the possible surfactants can be neutral, negatively charged, or zwitterionic but not positively charged. Neutral surfactants include (but not restricted to) polyoxyethylene alcohol's, polyoxyethylene isoalcohol, polyoxyethylene p-t-octyl phenol (Triton), polyoxyethylene nonylphenol, polyoxyethylene esters of fatty acids, polyoxyethylene sorbitol esters (Tween) and lipids. Negatively charged surfactants include (but not restricted to) di-(2-ethyl-hexyl) sodium sulfosuccinate (AOT), sodium dodecylsulfate (SDS), sodium dodecylsulfonate, and sodium dodecyl-N-sarcosinate. The zwitterionic surfactant could contain anionic and cationic groups on the alpha and omega positions of a long aliphatic chain. For zwitterionic surfactants that contain both anionic and cationic groups on the alpha and omega positions of a long aliphatic chain, complex formation should be done under acidic conditions so that the surfactant can have a positive charge that will interact with the nucleic acid. The anionic portion is neutralized by being protonated and therefore interacts with the non-aqueous phase. After formation of the complexes, the complexes are extracted into an aqueous solution containing a higher pH than the pH used to form the complexes.

Detailed Description

A biologically active compound is purified is when it is isolated from a mixture of other compounds. It is purified when its purity is increased where purity is defined as the percentage of the mixture containing the desired biologically active compound. Purification can also indicate a process where the purity of the desired compound is not increased but the other compounds within the mixture are changed. Purification also indicates the extraction of a biologic compound from one mixture or solution to another mixture or solution. This can include a process where the desired biologically active compound is moved from one solvent to another (also called extraction) or is solubilized within a solvent. A biologically active compound is a compound having the potential to react with biological components for gene therapy purposes. Pharmaceuticals, proteins, peptides, viruses, antigens, carbohydrates (and conjugates), lipids, saccharides, oligonucleotides, and nucleic acids are examples of biologically active compounds.

A surfactant refers to a compound that contains a polar group (hydrophilic) and a non-polar (hydrophobic) group on the same molecule. Cleavable surfactant refers to a surfactant in which the polar group may be separated from the nonpolar group by the removal of a chemical bond located between the two groups, or to a surfactant in which the polar or non-polar group or both may be chemically modified such that the detergent properties of the surfactant are destroyed. Cleavable also means that the surfactant is labile (able to be destroyed or that the detergent properties of the surfactant are able to be destroyed) and that its surfactant properties could be destroyed by other chemical processes than bond cleavage. A disulfide bond that is labile under physiological conditions means the disulfide bond is cleaved more rapidly than oxidized glutathione or any disulfide constructed from thiols in which one of the constituent thiols is more acidic, lower pKa, than glutathione or is activated by intramolecular attack by a free thiol. Constituent in this case means the thiols that are bonded together in the disulfide bond. The surfactant properties of a compound can be destroyed by chemical modification such as converting the polar group into a less polar group. This can be accomplished by a number of chemical modifications including (but not limited to) acylation, alkylation, elimination, reduction or oxidation, of an amine (or its salt), alcohol, diol (di-alcohol) or carboxylic acid groups, or by a multistep process in which several chemical modifications are conducted (for example oxidation of an alcohol to an aldehyde (ketone) followed by nucleophilic addition to the aldehyde (ketone) resulting in an alcohol followed by elimination of the alcohol (or a derivative of it).

Reverse (inverse) micelle refers to a surfactant with an internal aqueous pool. By "aqueous" we mean containing water, but can include buffers and salts. Non-aqueous solutions are made up of organic solvents such as hydrocarbon or halohydrocarbon. Buffers are made from a weak acid or weak base and their salts. Buffer solutions resist changes in pH when additional acid or base is added to the solution. Salts are ionic compounds that dissociate into cations and anions when dissolved in solution. Salts increase the ionic strength of a solution, and consequently decrease interactions between nucleic acids with other cations.

A reverse micelle is destroyed when the micelle no longer exists and the monophasic no longer exists. A reverse micelle is destroyed when the micelle is disrupted. In a preferred embodiment the reverse micelle is destroyed by chemically modifying the surfactant so that water in oil emulsion is destroyed and the phases separate. A

5 destructible reverse micelle is a reverse micelle that can be destroyed such that the water in oil emulsion is destroyed and the phases separate. A destructible reverse micelle can undergo a biological, chemical, or biochemical reaction such that the reverse micelle is destroyed. Biological, chemical, or biochemical reactions involve the formation or cleavage of ionic and/or covalent bonds. In a preferred embodiment the
10 destructible reverse micelle contains a surfactant that is cleavable, destroyable, or chemically modifiable. The surfactant can be a disulfide of the general formula A-S-S-B, in which chemical group A is a hydrophobic group and chemical group B is a hydrophilic group.

15 The present invention also relates to a method in which a biologically active compound is delivered to a cell comprising a step in which the biologically active compound is mixed with a biologically-labile surfactant. A biologically-labile surfactant is a surfactant in which the hydrophobic moiety is cleaved from the hydrophilic moiety by cellular processes or its surfactant properties are rendered inactive within the cell,
20 tissue or organism. Examples include surfactants that contain disulfide bonds that are labile within the cell, tissue, or organism.

A transfection reagent is a compound or compounds used in the prior art that bind(s) to or complex(es) with polynucleotides and mediates their entry into cells. The
25 transfection reagent also mediates the binding and internalization of polynucleotides into cells. Examples of transfection reagents include cationic liposomes and lipids, calcium phosphate precipitates, and polylysine complexes. At times, the transfection reagent has a net positive charge that binds to the polynucleotide's negative charge. The transfection reagent mediates binding of polynucleotides to cell via its positive
30 charge (that binds to the cell membrane's negative charge) or via ligands that bind to receptors in the cell. For example, cationic liposomes or polylysine complexes have net positive charges that enable them to bind to DNA.

Other vehicles are also used, in the prior art, to transfer genes into cells. These include complexing the polynucleotides on particles that are then accelerated into the cell. This is termed biolistic or gun techniques. Other methods include eletroporation in which a device is used to give an electric charge to cells. The charge increases the permeability of the cell.

The term "nucleic acid" is a term of art that refers to a polymer containing at least two nucleotides. "Nucleotides" contain a sugar deoxyribose (in DNA) or ribose (in RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and synthetic derivatives of purines and pyrimidines, or natural analogs. Nucleotides are the monomeric units of nucleic acid polymers. A "polynucleotide" is distinguished here from an "oligonucleotide" by containing more than 80 monomeric units; oligonucleotides contain from 2 to 80 nucleotides. The term nucleic acid includes deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA"). DNA may be in the form of anti-sense, plasmid DNA, parts of a plasmid DNA, vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives of these groups. RNA may be in the form of oligonucleotide RNA, tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, ribozymes, chimeric sequences, or derivatives of these groups. "Anti-sense" is a nucleic acid that interferes with the function of DNA and/or RNA. This may result in suppression of expression. Natural nucleic acids have a phosphate backbone, artificial nucleic acids may contain other types of backbones, nucleotides, or bases. These include PNAs (peptide nucleic acids), phosphothionates, and other variants of the phosphate backbone of native nucleic acids. In addition, DNA and RNA may be single, double, triple, or quadruple stranded. "Expression cassette" refers to a natural or recombinantly produced nucleic acid which is capable of expressing protein(s). A DNA expression cassette typically includes a promoter (allowing transcription initiation), and a sequence encoding one or more proteins. Optionally, the expression cassette may include trancriptional enhancers, non-coding sequences, splicing signals, transcription termination signals, and polyadenylation signals. An RNA expression cassette typically includes a translation

initiation codon (allowing translation initiation), and a sequence encoding one or more proteins. Optionally, the expression cassette may include translation termination signals, a polyadenosine sequence, internal ribosome entry sites (IRES), and non-coding sequences.

5

Delivery of a nucleic acid means to transfer a nucleic acid from a container outside a mammal to near or within the outer cell membrane of a cell in the mammal. The term “transfection” may be used, in general, as a substitute for the term “delivery,” or, more specifically, the transfer of a nucleic acid from directly outside a cell membrane to within the cell membrane. The transferred (or “transfected”) nucleic acid may contain an expression cassette. If the nucleic acid is a primary RNA transcript that is processed into messenger RNA, a ribosome translates the messenger RNA to produce a protein within the cytoplasm. If the nucleic acid is a DNA, it enters the nucleus where it is transcribed into a messenger RNA that is transported into the cytoplasm where it is translated into a protein. Therefore if a nucleic acid expresses its cognate protein, then it must have entered a cell. A protein may subsequently be degraded into peptides, which may be presented to the immune system.

Polypeptide refers to a linear series of amino acid residues connected to one another by peptide bonds between the alpha-amino group and carboxyl group of contiguous amino acid residues.

“Protein” refers herein to a linear series of greater than 2 amino acid residues connected one to another as in a polypeptide. A “therapeutic” effect of the protein in attenuating or preventing the disease state can be accomplished by the protein either staying within the cell, remaining attached to the cell in the membrane, or being secreted and dissociated from the cell where it can enter the general circulation and blood. Secreted proteins that can be therapeutic include hormones, cytokines, growth factors, clotting factors, anti-protease proteins (e.g., alpha1-antitrypsin), angiogenic proteins (e.g., vascular endothelial growth factor, fibroblast growth factors), anti-angiogenic proteins (e.g., endostatin, angiostatin), and other proteins that are present in the blood. Proteins on the membrane can have a therapeutic effect by providing a receptor for the cell to take up a protein or lipoprotein (e.g., low density lipoprotein

receptor). Therapeutic proteins that stay within the cell ("intracellular proteins") can be enzymes that clear a circulating toxic metabolite as in phenylketonuria. They can also cause a cancer cell to be less proliferative or cancerous (e.g., less metastatic), or interfere with the replication of a virus. Intracellular proteins can be part of the cytoskeleton (e.g., actin, dystrophin, myosins, sarcoglycans, dystroglycans) and thus have a therapeutic effect in cardiomyopathies and musculoskeletal diseases (e.g., Duchenne muscular dystrophy, limb-girdle disease). Other therapeutic proteins of particular interest to treating heart disease include polypeptides affecting cardiac contractility (e.g., calcium and sodium channels), inhibitors of restenosis (e.g., nitric oxide synthetase), angiogenic factors, and anti-angiogenic factors.

Biomolecule refers to peptides, polypeptides, proteins, enzymes, polynucleotides, oligonucleotides, viruses, antigens, carbohydrates (and congenates), lipids, and sacharides.

Enzymes are proteins evolved by the cells of living organisms for the specific function of catalyzing chemical reactions.

A chemical reaction can take place within the micelle and nucleic acid complex. A chemical reaction is defined as the formation or cleavage of covalent or ionic bonds. As a result of the chemical reaction a polymer can be formed. A polymer is defined as a compound containing more than two monomers. A monomer is a compound that can be attached to itself or another monomer and thus form a polymer. In one preferred embodiment the surfactant is polymerized by chain or step polymerization and then the surfactant properties are destroyed. This destruction of the surfactant properties could be accomplished by breaking a chemical bond and separating the hydrophilic and hydrophobic moities.

A chemical reaction can be used to attach a gene transfer enhancing signal to the nucleic acid complex. The gene transfer enhancing signal (or abbreviated as the Signal) is defined in this specification as a molecule that modifies the nucleic acid complex and can direct it or the nucleic acid to a cell location (such as tissue) or location in a cell (such as the nucleus) either in culture or in a whole organism. By modifying the

cellular or tissue location of the foreign gene, the expression of the foreign gene can be enhanced.

The gene transfer enhancing signal can be a protein, peptide, lipid, steroid, sugar,
5 carbohydrate, (non-expresssing) nucleic acid or synthetic compound. The gene transfer enhancing signals enhance cellular binding to receptors, cytoplasmic transport to the nucleus and nuclear entry or release from endosomes or other intracellular vesicles.

10 Nuclear localizing signals enhance the targeting of the gene into proximity of the nucleus and/or its entry into the nucleus. Such nuclear transport signals can be a protein or a peptide such as the SV40 large T ag NLS or the nucleoplasmin NLS. These nuclear localizing signals interact with a variety of nuclear transport factors such as the NLS receptor (karyopherin alpha) which then interacts with karyopherin beta.
15 The nuclear transport proteins themselves could also function as NLS's since they are targeted to the nuclear pore and nucleus.

Signals that enhance release from intracellular compartments (releasing signals) can cause DNA release from intracellular compartments such as endosomes (early and
20 late), lysosomes, phagosomes, vesicle, endoplasmic reticulum, golgi apparatus, trans golgi network (TGN), and sarcoplasmic reticulum. Release includes movement out of an intracellular compartment into cytoplasm or into an organelle such as the nucleus. Releasing signals include chemicals such as chloroquine, bafilomycin or Brefeldin A1 and the ER-retaining signal (KDEL sequence), viral components such as influenza
25 virus hemagglutinin subunit HA-2 peptides and other types of amphipathic peptides.

Cellular receptor signals are any signal that enhances the association of the gene with a cell. This can be accomplished by either increasing the binding of the gene to the cell surface and/or its association with an intracellular compartment, for example: ligands
30 that enhance endocytosis by enhancing binding the cell surface. This includes agents that target to the asialoglycoprotein receptor by using asialoglycoproteins or galactose residues. Other proteins such as insulin, EGF, or transferrin can be used for targeting. Peptides that include the RGD sequence can be used to target many cells. Chemical

groups that react with sulfhydryl or disulfide groups on cells can also be used to target many types of cells. Folate and other vitamins can also be used for targeting. Other targeting groups include molecules that interact with membranes such as lipids fatty acids, cholesterol, dansyl compounds, and amphotericin derivatives. In addition viral proteins could be used to bind cells.

A polynucleotide can be delivered to a cell in order to produce a cellular change that is therapeutic. The delivery of polynucleotides or other genetic material for therapeutic purposes (the art of improving health in an animal including treatment or prevention of disease) is gene therapy. The polynucleotides are coded to express a whole or partial protein, or may be anti-sense, and can be delivered either directly to the organism in situ or indirectly by transfer to a cell that is then transplanted into the organism. The protein can be missing or defective in an organism as a result of genetic, inherited or acquired defect in its genome. For example, a polynucleotide may be coded to express the protein dystrophin that is missing or defective in Duchenne muscular dystrophy. The coded polynucleotide is delivered to a selected group or groups of cells and incorporated into those cell's genome or remain apart from the cell's genome. Subsequently, dystrophin is produced by the formerly deficient cells. Other examples of imperfect protein production that can be treated with gene therapy include the addition of the protein clotting factors that are missing in the hemophilia's and enzymes that are defective in inborn errors of metabolism such as phenylalanine hydroxylase. A delivered polynucleotide can also be therapeutic in acquired disorders such as neurodegenerative disorders, cancer, heart disease, and infections. The polynucleotide has its therapeutic effect by entering the cell. Entry into the cell is required for the polynucleotide to produce the therapeutic protein, to block the production of a protein, or to decrease the amount of a RNA.

A therapeutic effect of the protein in attenuating or preventing the disease state can be accomplished by the protein either staying within the cell, remaining attached to the cell in the membrane or being secreted and dissociating from the cell where it can enter the general circulation and blood. Secreted proteins that can be therapeutic include hormones, cytokines, growth factors, clotting factors, anti-protease proteins (e.g. alpha-antitrypsin) and other proteins that are present in the blood. Proteins on the

membrane can have a therapeutic effect by providing a receptor for the cell to take up a protein or lipoprotein. For example, the low density lipoprotein (LDL) receptor could be expressed in hepatocytes and lower blood cholesterol levels and thereby prevent atherosclerotic lesions that can cause strokes or myocardial infarction.

- 5 Therapeutic proteins that stay within the cell can be enzymes that clear a circulating toxic metabolite as in phenylketonuria. They can also cause a cancer cell to be less proliferative or cancerous (e.g. less metastatic). A protein within a cell could also interfere with the replication of a virus.
- 10 The delivered polynucleotide can stay within the cytoplasm or nucleus apart from the endogenous genetic material. Alternatively, the polynucleotide could recombine (become a part of) the endogenous genetic material. For example, DNA can insert into chromosomal DNA by either homologous or non-homologous recombination.
- 15 Parenchymal cells are the distinguishing cells of a gland or organ contained in and supported by the connective tissue framework. The parenchymal cells typically perform a function that is unique to the particular organ. The term "parenchymal" often excludes cells that are common to many organs and tissues such as fibroblasts and endothelial cells within the blood vessels. In a liver organ, the parenchymal cells
- 20 include hepatocytes, Kupffer cells and the epithelial cells that line the biliary tract and bile ductules. The major constituent of the liver parenchyma are polyhedral hepatocytes (also known as hepatic cells) that presents at least one side to an hepatic sinusoid and apposed sides to a bile canaliculus. Liver cells that are not parenchymal cells include cells within the blood vessels such as the endothelial cells or fibroblast
- 25 cells.

- In striated muscle, the parenchymal cells include myoblasts, satellite cells, myotubules, and myofibers. In cardiac muscle, the parenchymal cells include the myocardium also known as cardiac muscle fibers or cardiac muscle cells and the cells of the impulse
- 30 connecting system such as those that constitute the sinoatrial node, atrioventricular node, and atrioventricular bundle. In a pancreas, the parenchymal cells include cells within the acini such as zymogenic cells, centroacinar cells, and basal or basket cells and cells within the islets of Langerhans such as alpha and beta cells. In spleen, thymus,

lymph nodes and bone marrow, the parenchymal cells include reticular cells and blood cells (or precursors to blood cells) such as lymphocytes, monocytes, plasma cells and macrophages.

In the nervous system which includes the central nervous system (the brain and spinal
5 cord) peripheral nerves, and ganglia, the parenchymal cells include neurons, glial cells, microglial cells, oligodendrocytes, Schwann cells, and epithelial cells of the choroid plexus. In the kidney, parenchymal cells include cells of collecting tubules and the proximal and distal tubular cells. In the prostate, the parenchyma includes epithelial cells. In glandular tissues and organs, the parenchymal cells include cells that produce
10 hormones. In the parathyroid glands, the parenchymal cells include the principal cells (chief cells) and oxyphilic cells. In the thyroid gland, the parenchymal cells include follicular epithelial cells and parafollicular cells. In the adrenal glands, the parenchymal cells include the epithelial cells within the adrenal cortex and the polyhedral cells within the adrenal medulla. In the parenchyma of the gastrointestinal tract such as the
15 esophagus, stomach, and intestines, the parenchymal cells include epithelial cells, glandular cells, basal, and goblet cells. In the parenchyma of lung, the parenchymal cells include the epithelial cells, mucus cells, goblet cells, and alveolar cells. In fat tissue, the parenchymal cells include adipose cells or adipocytes. In the skin, the parenchymal cells include the epithelial cells of the epidermis, melanocytes, cells of the
20 sweat glands, and cells of the hair root. In cartilage, the parenchyma includes chondrocytes. In bone, the parenchyma includes osteoblasts, osteocytes, and osteoclasts.

Intravascular refers to an intravascular route of administration that enables a polymer,
25 oligonucleotide, or polynucleotide to be delivered to cells more evenly distributed and more efficiently than direct injections. Intravascular herein means within an internal tubular structure called a vessel that is connected to a tissue or organ within the body of an animal, including mammals. Within the cavity of the tubular structure, a bodily fluid flows to or from the body part. Examples of bodily fluid include blood, lymphatic
30 fluid, or bile. Examples of vessels include arteries, arterioles, capillaries, venules, sinusoids, veins, lymphatics, and bile ducts. The intravascular route includes delivery through the blood vessels such as an artery or a vein. "Intracoronary" refers to an

intravascular route for delivery to the heart wherein the blood vessels are the coronary arteries and veins.

Permeability is defined herein as the propensity for macromolecules such as nucleic acids to move through vessel walls and enter the extravascular space. One measure of permeability is the rate at which macromolecules move through the vessel wall and out of the vessel. Another measure of permeability is the lack of force that resists the movement through the vessel wall and out of the vessel. Vessels contain elements that prevent macromolecules from leaving the intravascular space (internal cavity of the vessel). These elements include endothelial cells and connective material (e.g., collagen). High permeability indicates that there are fewer of these elements that can block the egress of macromolecules and that the spaces between these elements are larger and more numerous. In this context, high permeability enables a high percentage of nucleic acids being delivered to leave the intravascular space, while low permeability indicates that a low percentage of the nucleic acids will leave the intravascular space.

The permeability of a blood vessel can be increased by increasing the intravascular hydrostatic pressure. In a preferred embodiment, the intravascular hydrostatic pressure is increased by rapidly (from 1 seconds to 30 minutes) injecting a nucleic acid in solution into the blood vessel, which increases the hydrostatic pressure. In another preferred embodiment, hydrostatic pressure is increased by obstructing the outflow of the injection solution from the tissue for a period of time sufficient to allow delivery of a nucleic acid. Obstructing means to block or impede the outflow of injection fluid, thereby transiently (reversibly) blocking the outflow of the blood. Furthermore, rapid injection may be combined with obstructing the outflow in yet another preferred embodiment. For example, an afferent vessel supplying an organ is rapidly injected while the efferent vessel draining the tissue is blocked transiently (e.g., by ligation, or by an inflated intravascular balloon). The efferent vessel (also called the venous outflow or tract) draining outflow from the tissue is partially or totally clamped for a period of time sufficient to allow delivery of a nucleic acid. In the reverse, an efferent vessel is injected while the corresponding afferent vessel is occluded.

An administration route involving the mucosal membranes is meant to include nasal, bronchial, inhalation into the lungs, or via the eyes.

Transdermal refers to application to mammal skin in which drug delivery occurs by
5 crossing the dermal layer.

Crosslinking refers to the chemical attachment of two or more molecules with a bifunctional reagent. A bifunctional reagent is a molecule with two reactive ends. The reactive ends can be identical as in a homobifunctional molecule, or different as in a
10 heterobifunctional molecule.

Electrostatic interactions are the non-covalent association of two or more substances due to attractive forces between positive and negative charges.

15 Amphipathic compounds have both hydrophilic (water-soluble) and hydrophobic (water-insoluble) parts.

A polycation is a polymer containing a net positive charge, for example poly-L-lysine hydrobromide. The polycation can contain monomer units that are charge positive,
20 charge neutral, or charge negative, however, the net charge of the polymer must be positive. A polycation also can mean a non-polomeric molecule which contains two or more positive charges. A polyanion is a polymer containing a net negative charge, for example polyglutamic acid. The polyanion can contain monomer units that are charge negative, charge neutral, or charge positive, however, the net charge on the polymer
25 must be negative. A polyanion can also mean a non-polomeric molecule that contains two or more negative charges. The term polyion includes polycation, polyanion, zwitterionic polymers, and neutral polymers. The term zwitterionic refers to the product (salt) of the reaction between an acidic group and a basic group that are part of the same molecule.

30 Mixing means the method of interdispersing two or more solvents, or solvent(s) and solute(s). Sonication and agitation are forms of mixing. Solvent refers to a material in the liquid phase that can be used to solubilize (dissolve) a compound. Solute refers to a compound dissolved in a solvent.

Hydrocarbon means containing carbon and hydrogen atoms; and halohydrocarbon means containing carbon, halogen (F, Cl, Br, I), and hydrogen atoms.

5 Alkyl means containing sp³ hybridized carbon atoms; alkenyl means containing two or more sp² hybridized carbon atoms; alkynyl means containing two or more sp hybridized carbon atoms; aralkyl means containing one or more aromatic ring(s) in addition containing sp³ hybridized carbon atoms; aralkenyl means containing one or more aromatic ring(s) in addition to containing two or more sp² hybridized carbon
10 atoms; aralkynyl means containing one or more aromatic ring(s) in addition to containing two or more sp hybridized carbon atoms; steroid includes natural and unnatural steroids and steroid derivatives. A steroid derivative means a sterol, a sterol in which the hydroxyl moiety has been modified (for example, acylated), or a steroid hormone, or an analog thereof; carbohydrates include natural and unnatural sugars (for example glucose), and sugar derivatives (a sugar derivative means a system in which
15 one or more of the hydroxyl groups on the sugar moiety has been modified (for example acylated), or a system in which one or more of the hydroxyl groups is not present); polyoxyethylene means a polymer having two to six (n=2-6) ethylene oxide units $-(CH_2CH_2O)_n-$ or a derivative thereof; and R not identified by number is meant
20 to be any compatible group, for example alkyl, alkenyl, alkynyl, aralkyl, aralkenyl, or aralkynyl, and can include heteroatoms (N, O, S), and carbonyl groups.

Hydrophilic groups indicate in qualitative terms that the chemical moiety is water-preferring. Typically, such chemical groups are water soluble, and are hydrogen bond
25 donors or acceptors with water. Examples of hydrophilic groups include compounds with the following chemical moieties carbohydrates; polyoxyethylene, peptides, oligonucleotides and groups containing amines, amides, alkoxy amides, carboxylic acids, sulfurs, or hydroxyls. Hydrophobic groups indicate in qualitative terms that the chemical moiety is water-avoiding. Typically, such chemical groups are not water
30 soluble, and tend not to hydrogen bond. Hydrocarbons are hydrophobic groups.

The terms "therapeutic" and "therapeutic results" are defined in this application as levels of gene products, including reporter (marker) gene products, which indicate a

reasonable expectation of gene expression using similar compounds (other nucleic acids including other genes), at levels considered sufficient by a person having ordinary skill in the art of gene therapy. For example: Hemophilia A and B are caused by deficiencies of the X-linked clotting factors VIII and IX, respectively. Their clinical course is greatly influenced by the percentage of normal serum levels of factor VIII or IX: < 2%, severe; 2-5%, moderate; and 5-30% mild. This indicates that in severe patients only 2% of the normal level can be considered therapeutic. Levels greater than 6% prevent spontaneous bleeds but not those secondary to surgery or injury. A person having ordinary skill in the art of gene therapy would reasonably anticipate therapeutic levels of expression of a gene specific for a disease based upon sufficient levels of marker gene results. In the Hemophilia example, if marker genes were expressed to yield a protein at a level comparable in volume to 2% of the normal level of factor VIII, it can be reasonably expected that the gene coding for factor VIII would also be expressed at similar levels.

There are three types of reporter (marker) gene products that are expressed from reporter genes. The reporter gene/protein systems include:

- a) Intracellular gene products such as luciferase, β -galactosidase, or chloramphenicol acetyl transferase. Typically, they are enzymes whose enzymatic activity can be easily measured.
- b) Intracellular gene products such as β -galactosidase or green fluorescent protein which identify cells expressing the reporter gene. On the basis of the intensity of cellular staining, these reporter gene products also yield qualitative information concerning the amount of foreign protein produced per cell.

Secreted gene products such as growth hormone, factor IX, or alpha1-antitrypsin are useful for determining the amount of a secreted protein that a gene transfer procedure can produce. The reporter gene product can be assayed in a small amount of blood.

Examples

Compound Preparation:

Synthesis of (-D-Glucopyranosyl dodecane disulfide

To a solution of dodecane thiol (1.00 mL, 4.17 mmol, Aldrich) in 20 mL CHCl₃ was added sulfuryl chloride (0.74 mL, 9.18 mmol, Aldrich), and the resulting mixture was stirred at room temperature for 18 h. Removal of solvent (aspirator), afforded dodecansulfenyl chloride that was determined to be sufficiently pure by ¹H NMR.

5

To a solution of dodecansulfenyl chloride (213 mg, 0.899 mmol) in 2.7 mL acetonitrile was added (-D-thioglucose sodium salt (200. mg, 0.917 mmol, Aldrich) and 15-crown-5 (0.18 mL, 0.899 mmol, Aldrich). The resulting mixture was stirred at ambient temperature for 3 h, and the solvent removed (aspirator). The residue was triturated with CHCl₃ and filtered. The residue was purified by flash column chromatography on silica gel (0-5% MeOH in CH₂Cl₂). Crystallization (EtOAc) afforded 85 mg (24 %) of a fine white solid.

10

I. Example 1: Synthesis of β -D-Glucopyranosyl dodecane disulfide

15

To a solution of dodecane thiol (1.00 mL, 4.17 mmol, Aldrich Chemical Company) in 20 mL CHCl₃ was added sulfuryl chloride (0.74 mL, 9.18 mmol, Aldrich Chemical Company), and the resulting mixture was stirred at room temperature for 18 h. Removal of solvent (aspirator), afforded dodecansulfenyl chloride that was determined to be sufficiently pure by ¹H NMR.

20

To a solution of dodecansulfenyl chloride (213 mg, 0.899 mmol) in 2.7 mL acetonitrile was added 1-thio-β-D-glucose sodium salt hydrate (200. mg, 0.917 mmol, Aldrich Chemical Company) and 15-crown-5 (0.18 mL, 0.899 mmol, Aldrich Chemical Company). The resulting mixture was stirred at ambient temperature for 3 h, and the solvent removed (aspirator). The residue was triturated with CHCl₃ and filtered. The residue was purified by flash column chromatography on silica gel (0-5% MeOH in CH₂Cl₂). Crystallization (EtOAc) afforded 85 mg (24 %) of β -D-glucopyranosyl dodecane disulfide as a fine white solid.

25

Example 2: Demonstration of Micelle Formation with β -D-Glucopyranosyl dodecane disulfide, and Micelle Destruction with Dithiothreitol:

To a solution of β -D-Glucopyranosyl dodecane disulfide (10 mg) in 1 mL CD_3Cl was added 1 mL H_2O . The sample was rapidly mixed resulting in a thick white emulsion. After 18 h, the organic and aqueous layers were emulsified to approximately 95%.

5 After 4 d, the organic and aqueous layers remained emulsified to approximately 70%.

To a 1 mL portion of the emulsion was added 60 μg of dithiothreitol (Aldrich Chemical Company), and the solution was mixed. After 30 min, the emulsion had cleared. 5,5'-Dithiobis(2-nitrobenzoic acid) (1mg, Aldrich Chemical Company) was added, resulting in a yellow solution, verifying the presence of free sulfide. Analysis
10 also indicated the presence of dodecane thiol and 1-thio- β -D-glucose by TLC.

Example 3. Solubilization of PCILuc DNA in Reversed Micelles.

Procedure:

15 PCILuc DNA (pDNA) (Zhang, G., Vargo, D., Budker, V., Armstrong, N., Knechtle, S., Wolff, J. A. Human Gene Therapy, 8, 1763-1772, 1997) (11 μg) was taken up in a solution (3-67 μL) of HEPES (25 mM, pH 7.8) and EDTA (0.5 mM).

Polyoxyethylene(4) lauryl ether (Brij 30) (1.2 mL, Aldrich Chemical Company) was taken up in 2,2,4-trimethylpentane (TMP) (8.8 mL, Aldrich Chemical Company). To
20 the Brij 30/TMP solution (0.7 mL) was added the pDNA in buffer (3-67 μL). The mixtures were shaken (2 min) resulting in clear solutions. After 10 min the turbidity was determined utilizing a fluorescence spectrophotometer (Hitachi, model F3010, extinction/emission wavelength of 529 nm). W_0 is defined as the molar ratio of water to surfactant.

25

Results:

$\text{H}_2\text{O}(\mu\text{L})$	W_0	Turbidity (529 nm)
0	0	19
3	0.72	49
7	1.68	63

30

12	2.87	63
17	4.07	82
5 27	6.46	2764
47	11.25	1565
10 67	16.04	214

Analysis:

At 20 °C, the pDNA solution when added to the Brij 30/TMP forms inversed micelles for an aqueous content of up to $W_0=4$. For a solution of Brij 30 in dodecane the hydrophile-lipophile balance (HLB) temperature has been determined to be approximately 29.2 °C (Kunieda, H. Langmuir 7,1915, 1991). For the present system it was shown that below 29.2 °C, w/o microemulsion are present for a W_0 of less than 10. By increasing the water content, one obtains a two-phase system and then a lamellar phase. In the case of Brij 30 in TMP, a two-phase system appears at W_0 of more than 6 and a lamellar phase at W_0 of more than 11.

Example 4. Determination of the Size of PCILuc DNA Contained in Inversed Micelles.

25 Part A. Centrifugation.

Procedure:

PCILuc DNA (pDNA) (Zhang, G., Vargo, D., Budker, V., Armstrong, N., Knechtle, S., Wolff, J. A. Human Gene Therapy, 8, 1763-1772, 1997) (36 µg) was taken up in a solution of HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) (10 µL, 20 µL, 30 µL, and 50 µL). The resulting solutions were added to a mixture of Polyoxyethylene(4) lauryl ether (Brij 30) (Aldrich Chemical Company)/ 2,2,4-trimethylpentane (TMP) (Aldrich Chemical Company) (1 mL, 1:7.3 v/v) and agitated. The UV adsorption was determined (Perkin Elmer, UV/VIS Spectrophotometer, model Lambda 6) against 10

μL of HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) buffer in Brij 30/TMP (1 mL, 1:7.3 v/v). The samples were centrifuged 5 min at 15000 rpm and the UV adsorption was again determined.

5 Results:

Conditions	W_0	A_{260} before centrifugation	A_{260} after centrifugation
A. DNA in buffer		- 1.07	1.07
10 μL	1.68	1.07	1.11
10 20 μL	3.36	0.99	1.14
30 μL	5.04	0.97	1.01
15 50 μL	8.39	2.44	ND ^a

^a UV absorption not determined. Solution was two-phase.

Analysis:

20 At 20 °C, micelles that contain pDNA (up to $W_0=5$) are small enough to stay in solution in the course of centrifugation. For these solutions, no change in the UV absorption spectra was recorded as compared to the UV absorption of pDNA in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM).

25 **Part B. Particle Size of Micelles Without PCILuc DNA.**

Procedure:

A solution (5 - 50 μL) of HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) was added to a mixture of Brij 30/TMP (1 mL, 1:7.3 v/v) and agitated (2 min). The samples were centrifuged (1 min) at 12000 rpm and the size of micelles measured (Particle Sizer,
30 Brookhaven Instrument Corporation).

Results:

Volume of buffer (μL)	W_0	Size nm
------------------------------------	-------	---------

	0	0	1.3
	5	0.84	2.9
5	10	1.68	3.4
	20	3.35	5.1
10	30	5.04	9.7
	50	8.39	indefinite

Analysis:

- 15 The size of the micelles changes proportionally as the water content increases, from 1.3 nm for "dry" micelles to 9.7 nm for micelles with $W_0=5$. At a higher water content, a two-phase system is present.

Part C. Particle Size of Micelles Containing PCILuc DNA.

20 Procedure:

A solution pDNA in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) was added to a mixture of Brij 30/TMP (1 mL, 1:7.3 v/v) and agitated (2 min) to form micelles with a W_0 of 3.35. The samples were centrifuged (1 min) at 12000 rpm and the size of micelles was measured (Particle Sizer, Brookhaven Instrument Corporation).

25

Results:

	B. DNA ng	Small micelles (nm)	Large micelles (nm)
	0	5.1	-
30	40	4.0	16.2
	80	4.7	48.7

120	4.7	62.8
160	4.4	51.7

5

Analysis:

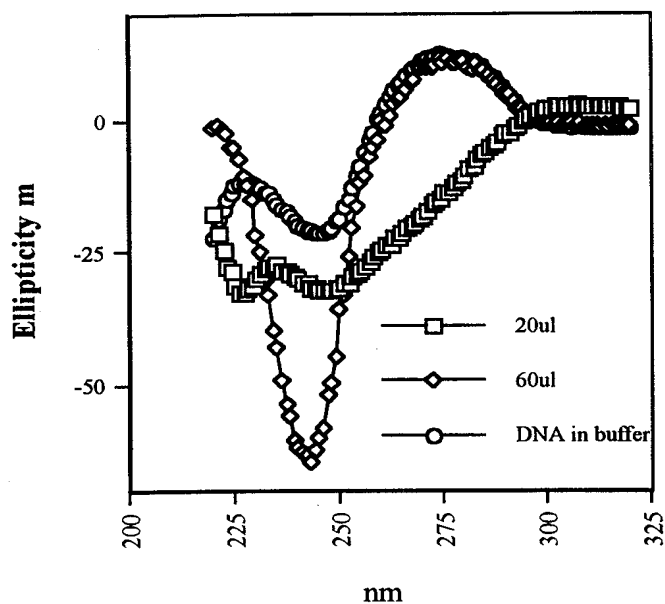
Two types of micelles appear to be present in the samples. There are small, "empty" micelles, and large pDNA containing micelles. It appears that the size of micelles containing pDNA increases as the concentration of pDNA increases. The micelle
 10 appears to be saturated at a size of 50-60 nm.

Example 5: Conformation of PCILuc DNA in Inverse Micelles.15 **Procedure:**

pDNA (60 μ g) was taken up in 10 mM potassium phosphate buffer at pH 7.5 (20 μ L and 60 μ L). The pDNA solutions were added to a mixture of Brij 30/TMP (1 mL, 1:7.3 v/v) and agitated (2 min). The circular dichroism spectra were measured for each sample (cell length = 0.5 cm, Spectropolarimeter 62DS, Avive Associates) at 30 $^{\circ}$ C
 20 against control samples prepared without the pDNA (the ellipticity value for the control samples were subtracted from the experimental samples).

Results:

There are shifts in the position of both the positive and negative bands and in the
 25 position of the cross-over point for the 20 μ L pDNA solution ($W_0=3.35$). Spectra that are similarly shifted are broadly defined as -spectra, and are attributed to a condensed form of pDNA. In contrast the spectra of the 60 μ L pDNA solution ($W_0=10.05$) resembles the spectra of DNA in buffer alone in respect to cross-over point. However
 30 this spectra is characterized by an increase in the intensity of the negative band (maximum at 240 nm).



5 **Example 6: PCILuc DNA Condensation.**

Part A. Ethidium Bromide.

Procedure:

A solution of pDNA in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) (3-67 μ L)
10 containing ethidium bromide (0.9 μ g, Sigma Chemical Company) was added to a
mixture of Brij 30/TMP (0.7 mL, 1:7.3 v/v) and agitated. After 4 h at ambient
temperature, the samples were assayed utilizing a fluorescence spectrophotometer
(Hitachi, Model F-3010), with an excitation wavelength of 525 nm and an emission
wavelength of 595 nm.

15

Results:

	Volume (μL)	W_0	$I/I_{\text{max}}*100$
	3	0.72	15
5	7	1.68	13
	12	2.87	12
10	17	4.07	12.5
	27	6.46	23
	47	11.25	35
15	67	16.04	51

Analysis:

The pDNA in reverse micelles of up to $W_0=4$ is condensed. Additionally, some level of condensation is shown for micelles up to $W_0=16$.

Part B: Determination of Rhodamine Labeled DNA Condensation in a Reverse Micelle.

Procedure:

pDNA was modified to a level of 1 Rhodamine per 100 bases using Mirus' LabelIt® Rhodamine kit (Rhodamine Containing DNA Labeling Reagent, Mirus Corporation). The modified pDNA (2.5 μg) was solubilized in different volumes of HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) and added to a solution of Brij 30/TMP (0.7 mL, 1:7.3 v/v), and agitated. The fluorescence was determined using a fluorescence spectrophotometer (Hitachi, Model F-3010), at an excitation wavelength of 591 nm, and an emission wavelength of 610 nm.

Results:

	Buffer Volume (μ L)	W_0	$(I_{610} \text{ sample}/I_{610} \text{ DNA in buffer}) * 100$
	2	0.48	104
5	4	0.96	80
	5	1.2	34
	10	2.39	31
10	12	2.87	24
	15	3.59	33
15	22	5.26	32
	32	7.66	65
	42	10.0	106
20	52	12.45	93
	62	14.84	78

25 It should be noted that around $W_0=10$ turbidity has significant contribution in fluorescence. The assay indicates that under low water conditions, pDNA does not appear to be condensed. As the amount of water in the system is increased, the fluorescence results indicate that pDNA is condensed within the w/o microemulsion.

30 II. Example 7: pDNA Condensation in Reverse Micelles

Procedure:

pDNA was modified to a level of 1 Rhodamine per 100 bases using standard procedures (LabelIt[®], Mirus Corporation). Labeled pDNA (various amounts) was

taken up in HEPES (25 mM, pH 7.8) EDTA (0.5 mM) (various amounts) and was mixed with unmodified pDNA (various amounts) to afford 2.5 µg total of pDNA. The resulting solution was added to Brij 30/TMP (0.7 mL, 1:7.3 v/v) and the fluorescence was determined using a fluorescence spectrophotometer (Hitachi, Model F-3010), at an excitation wavelength of 591 nm, and an emission wavelength of 610 nm.. For comparison, the fluorescence was also determined for the similar ratios of Rh-labeled pDNA/pDNA containing 2 mM spermidine (Sigma Chemical Company) in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) (0.7 mL).

Results:

% Rh-DNA	% of Fluorescence quenching			
	W ₀ =2.39	W ₀ =3.59	W ₀ =7.18	2mM Sp.
100	68.8	61.2	41.3	69.8
76	65.9	57.5	33.1	61
51	59	52.2	30	48
26	55.5	50.4	28.3	26.1

Analysis:

- The fluorescence data indicates a relatively weak affect of Rh-labeled pDNA dilution by unlabeled pDNA. On the other hand, in the samples containing spermidine, a strong effect of the Rh-pDNA dilution by unlabeled DNA is shown. In reverse micelles, the pDNA condensation starts from monomolecular condensation and therefore show little effect by the dilution protocol. However, in the spermidine containing systems (non-micellular) the strong effect indicates that condensation is multimolecular.

III. Example 8: Transmission Electron Microscope Assay

Procedure:

- A drop of Poly-L-lysine (PLL) (30-70KD, Sigma Chemical Company) in water (concentration of 10 mg/mL) was placed on a covered EM grid. The solution was removed, and the grid was dried. A drop of 2,2,4-trimethylpentane (TMP) (Aldrich

Chemical Company) in various amounts of HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) both with and without PCILuc DNA (pDNA) (7 µg/mL TMP) was placed on the grid. After 5 min, the solution was removed and the grid was washed with TMP (3 x) and water (1 x), and then stained with Uranyl Acetate.

5

Results:

Samples containing 20 or 60 µL of HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) in TMP (1 mL) failed to show any structures. A sample containing pDNA (7 µg) in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) in TMP (1 mL) also failed to show any structures. A sample containing pDNA in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) (20 µL) and TMP (1 mL) demonstrated ring like structures with an external diameter of 59.8 ± 12.5 nm and an internal diameter of 32.9 ± 12.1 nm. A sample of pDNA in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) (60 µL) and TMP (1 mL) demonstrated long threads with a 7-12 nm diameter. The volume of the terroid ring

$V = (\pi^{2/4})(R_{out} - R_{in})^2(R_{out} + R_{in})$ equal $41 \cdot 10^3 \text{ nm}^3$. The volume of "dry" PCILuc DNA is $6.4 \cdot 10^3 \text{ nm}^3$. With consideration of packing parameter every toroid therefore contains five pDNA's.

10

15

Example 9: PCILuc DNA /Labeled Poly-L-Lysine Interaction.

Procedure:

To Poly-L-lysine (PLL) (4 mg, Sigma Chemical Company) in potassium phosphate buffer (pH 8, 0.1 mL) was added 7-Chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) (0.4 mg, Sigma Chemical Company). The solution was heated at 37°C for 2 h, cooled, and purified by gel-filtration on Sephadex G-25. The fluorescence was determined (Hitachi, model F-3010, excitation wavelength = 466 nm, emission wavelength = 540 nm), and the level of modification was estimated to be 5%. To the NBD-PLL (5 µg) in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) (1 mL), was added pDNA, and the fluorescence was again determined.

25

Results:

30

pDNA µg	0	1	2	4	6
I ₅₄₀	41	27	21	17	16

Analysis:

The interaction of the NBD-PLL with pDNA was shown to substantially decrease the quantum yield of fluorescence.

5

Example 10: PCILuc DNA/Polycation Interaction in a Reverse Micelle.**Procedure:**

NBD-PLL (example 9) was mixed with Polyoxyethylene(4) lauryl ether (Brij 30) (Aldrich Chemical Company)/ 2,2,4-trimethylpentane (TMP) (Aldrich Chemical
 10 Company) (1:7.3 v/v), and then mixed with an equal volume of Brij 30/TMP (1:7.3 v/v) that contained either HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) or HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) with pDNA (various amounts) After 10 min at ambient temperature, the fluorescence was determined for each sample.

Conditions	I ₅₄₀
0.5 mL TMPD with 5µg NBD-PLL in 20 µL buffer + 0.5 mL TMPD with 20 µL buffer	87
0.5 mL TMPD with 5µg NBD-PLL in 20 µL buffer + 0.5 mL TMPD with 3.7 µg DNA in 20 µL buffer	64
0.5 mL TMPD with 5µg NBD-PLL in 20 µL buffer + 0.5 mL TMPD with 11.1 µg DNA 20 µL buffer	38

15

Analysis:

The results from the fluorescence study indicate that pDNA in reverse micelles can interact with PLL also in reverse micelles.

20 IV. Example 11: PCILuc DNA/Crosslinked Polycation Interaction**Procedure:**

To a solution of pDNA (35 µg) in HEPES (25 mM, pH 7.8), EDTA (0.5 mM), and NaCl (100 mM) (24 µL) was added Polyoxyethylene(4) lauryl ether (Brij 30) (Aldrich Chemical Company)/ 2,2,4-trimethylpentane (TMP) (Aldrich Chemical
 25 Company) (510 µL, 1:7.3 v/v). Poly-L-lysine (PLL) (95 µg, Sigma Chemical Company) in HEPES (25 mM, pH 7.8), EDTA (0.5 mM), and NaCl (100 mM) (12 µL) was added to Brij 30/TMP (290 µL, 1:7.3 v/v). The resulting solutions were

mixed and heated to 40 °C for 30 min at which time Dimethyl 3,3'-dithiobispropionimidate-2HCl (DTBP, Pierce Chemical Company) in DMSO (various amounts of a 29.5 mg/mL solution) were added. The solution was heated to 40 °C for 25 min at which time HEPES (25 mM, pH 7.8), EDTA (0.5 mM), and NaCl (100 mM) (200 µL) was added, followed by EtOH (50 µL) and EtOAc (0.5 mL). After mixing and centrifugation, the aqueous layer was washed with EtOAc (2 x 1 mL) and Ether (2 x 1 mL). The samples were spun (5 min, 12000 rpm) and dialyzed for 16 h against HEPES (25 mM, pH 7.8) and NaCl (100 mM). The UV absorption was determined (Perkin Elmer UV/VIS Spectrophotometer, Model Lambda 6). A solution of TO6 (Zeng, Z., Clark, S. M., Mathies, R. A., Glazer, A. N. Analytical Biochemistry, 252, 110-114, 1997) (2 µL, 0.5 mg/mL in water) was added and the fluorescence was determined (Hitachi, Model F-3010, excitation wavelength = 509 nm, emission wavelength = 540 nm).

Results:

#	Amount of DTBP µl	% DNA recovery	Fluorescence
35 µg DNA(no treatment)	-	100	120.4
1	0	3	0.275
2	3	14	1.76
3	6	19	3.07
4	12	24	4.02

Analysis:

The results indicate that the pDNA-PLL complex can be partly extracted from reverse micelles after the PLL has been crosslinked with DTBP. The pDNA in the extracted complexes is compacted because it does not interact with the fluorescent intercalator TO6.

Example 12: PCILuc DNA/Polyethylenimine Complexes in Reverse Micelles.

Procedure:

pDNA was labeled as above (LabelIt[®], Mirus Corporation). Labeled pDNA (14 µg) was taken up in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) (various amounts) and added to Polyoxyethylene(4) lauryl ether (Brij 30) (Aldrich Chemical Company)/2,2,4-trimethylpentane (TMP) (Aldrich Chemical Company) (1 mL, 1:7.3 v/v). The

fluorescence and turbidity of each sample was determined. Polyethylenimine (PEI) (30 μ g, Sigma Chemical Company) in HEPES (25 mM, pH 7.8) and EDTA (0.5 mM) (3 μ L) was added to each sample. After 30 min the fluorescence and turbidity of each sample was determined.

5

Results:

	No PEI		With PEI	
	I ₆₁₀	Turbidity	I ₆₁₀	Turbidity
DNA in buffer	28.45	31	8.7	76
W ₀ =0.67	14.8	105	11.5	164
1.51	9.7	103	10.2	144
2.35	11.0	85	11.8	114
4.03	18.3	105	15.9	137
5.71	26.0	182	18.0	217
9.06	31.6	4200	17.8	4734

Analysis:

- 10 The decrease in fluorescence after the addition of PEI indicates that PEI was within the same micelle as the DNA and was bound to it.

- 15 The foregoing examples are considered as illustrative only of the principles of the invention. Further, since numerous modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and operation shown and described. Therefore, all suitable modifications and equivalents fall within the scope of the invention.

We Claim:

- 1) A complex for delivery to a cell, comprising: inserting a nucleic acid into a reverse micelle.
- 2) The complex of claim 1 wherein the nucleic acid is compacted.
- 3) The complex of claim 2 wherein the nucleic acid interacts with a compound within the reverse micelle.
- 4) The complex of claim 2 wherein the nucleic acid is extractable from the reverse micelle.
- 5) The complex of claim 4 wherein the complex further comprises a surfactant.
- 6) The complex of claim 5 wherein the surfactant contains a disulfide bond.
- 7) The complex of claim 6 wherein the surfactant contains a hydrophobic portion and a hydrophilic portion.
- 8) The complex of claim 3 wherein the compound is a compacting agent to further compact the nucleic acid.
- 9) The complex of claim 3 wherein the compound comprises a delivery enhancing ligand.
- 10) The complex of claim 2 wherein the reverse micelle is coated with a layer.
- 11) A process for delivering a complex to a cell, comprising: inserting a nucleic acid into a reverse micelle.
- 12) The process of claim 11 wherein a delivery enhancing compound is added to the complex.

- 13) The process of claim 12 wherein the compound comprises a compacting agent.
- 14) The process of claim 12 wherein the compound comprises a polymer.
- 15) The process of claim 11 wherein the nucleic acid is removable from the reverse micelle.
- 16) The process of claim 15 wherein removing the nucleic acid comprises:
- a) cleaving the reverse micelle; and,
 - b) extracting the nucleic acid.
- 17) The process of claim 16 further comprising separating phases formed by mixing the nucleic acid with a molecule.
- 18) The process of claim 16 wherein cleaving the reverse micelle includes: mixing the nucleic acid with a separator selected from the group consisting of a hydrocarbon and a halohydrocarbon.
- 19) The process of claim 18 wherein the halohydrocarbon comprises a surfactant having a hydrophobic portion and a hydrophilic portion.
- 20) The process of claim 18 wherein the surfactant contains a disulfide bond.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/16107

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/10, 15/87, 15/88; C07H 21/04

US CL : 435/455, 458; 424/450; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/455, 458; 424/450; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, WEST, Medline, Biotech

Search terms: reverse micelle, liposome, disulfide bond, surfactant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,635,487 A (WOLFF et al.) 03 June 1997, columns 6-7.	1-20
A	BOUSSIF et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and <i>in vivo</i> : Polyethylenimine. Proceedings of the National Academy of Sciences USA. August 1995, Vol. 92, pages 7297-7301, especially page 7298.	1-20
A	CHONN et al. Recent advances in liposomal drug-delivery systems. Current Biology. 1995, Vol. 6, pages 698-708, especially Figure 1.	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

14 OCTOBER 1999

Date of mailing of the international search report

04 NOV 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DAVID GUZO

Telephone No. (703) 308-0196